EAST Search History

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	0	09/403861.pn.	US-PGPUB; USPAT; EPO; DERWENT	OR	OFF	2006/09/04 10:55
L2	2	"09403861"	US-PGPUB; USPAT; EPO; DERWENT	OR	OFF	2006/09/04 10:55
L3	2	"6833348".pn.	US-PGPUB; USPAT; EPO; DERWENT	OR	OFF	2006/09/04 10:56

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Day : Monday
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Continuity Information for 10/630926

Parent Data

10630926
is a continuation in part of 09403861
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Child Data

No Child Data

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Application Number Information

Application Number: **09/403861** Order This File Assignments
Examiner Number: **76570 / EPPS FORD, JANET**
Filing or 371(c) Date: **02/11/2000**
Group Art Unit: **1635** **IFW IMAGE**
Effective Date: **02/11/2000**
Class/Subclass: **514/002.000**
Application Received: **02/11/2000**
Lost Case: **NO**
Patent Number: **6833348**
Interference Number:
Issue Date: **12/21/2004**
Unmatched Petition: **NO**
Date of Abandonment: **00/00/0000**
L&R Code: Secrecy Code:**1**
Attorney Docket Number: **RICCARDI=1**
Third Level Review: **NO**
Secrecy Order: **NO**
Status: **150 /PATENTED CASE**
Status Date: **12/02/2004**
Confirmation Number: **7791**
Oral Hearing: **NO**
Title of Invention: **INTRACELLULAR GLUCOCORTICOID-INDUCED LEUCINE ZIPPERS MODULATORS OF APOPTIC CELL DEATH PATHWAYS**

Bar Code	PALM Location	Location Date	Charge to Loc	Charge to Name	Employee Name	Location
09403861CA	9200	02/14/2005	No Charge to Location	No Charge to Name	MASSILLON,PRADIEU	

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-continued

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(D) OTHER INFORMATION:/note= "the N-terminus is modified by an acetyl group; the C-terminus is modified with CH2OC(O)-[2,6-(CF3)2]Ph"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Tyr Val Ala Asp

What is claimed is:

1. A glucocorticoid-induced leucine-zipper family related (GILR) protein capable of inhibiting apoptosis and stimulating lymphocyte activity, wherein said GILR protein:

(a) is encoded by the nucleotide sequence of SEQ ID NO: 1; or

(b) contains no more than five amino acid changes from the amino acid sequence of SEQ ID NO:2, each of said changes being either alternative conservative substitutions within one of the following five groups of amino acid residues:

(1) Ala, Ser, Thr, Pro, Gly;

(2) Asp, Asn, Glu, Gln;

(3) His, Arg, Lys;

(4) Met, Leu, Ile, Val, Cys; and

(5) Phe, Tyr, Trp

or deletion of one or more of residues 123, 124, and 125 of SEQ ID NO:2.

2. A composition for the inhibition of apoptosis in cells or for stimulating lymphocyte activation, comprising, as an active ingredient, the GILR protein of claim 1.

3. A chemically modified GILR protein of claim 1, wherein said GILR protein of claim 1 is chemically modified by being conjugated or complexed with molecules facilitating or enhancing the transport of said GILR protein across cell membrane and wherein the chemically modified GILR protein has the same or higher biological activity as said GILR protein.

4. A composition for the inhibition of apoptosis in cells or for stimulating lymphocyte activation, comprising, as an active ingredient, the chemically modified GILR protein of claim 3.

5. The GILR protein of claim 1, which is encoded by the nucleotide sequence of SEQ ID NO:1.

6. The GILR protein of claim 1, wherein said no more than five amino acid changes from the amino acid sequence of SEQ ID NO:2 are present at amino acid residue positions selected from the group consisting of residue positions 22, 50, 75, 84, 112, 122, 123, 124, 125, 127, and 128 of SEQ ID NO:2.

7. The GILR protein of claim 1, which contains no more than three amino acid changes from the amino acid sequence of SEQ ID NO:2.

8. The GILR protein of claim 7, wherein said no more than three amino acid changes from the amino acid sequence of SEQ ID NO:2 are present at amino acid residue positions selected from the group consisting of residue positions 22, 50, 75, 84, 112, 122, 123, 124, 125, 127, and 128 of SEQ ID NO:2.

9. The GILR protein of claim 1, which contains a single amino acid change from the amino acid sequence of SEQ ID NO:2.

10. The GILR protein of claim 9, wherein said single amino acid change from the amino acid sequence of SEQ ID NO:2 is present at an amino acid position selected from the group consisting of residue positions 22, 50, 75, 84, 112, 122, 123, 124, 125, 127, and 128 of SEQ ID NO:2.

11. A glucocorticoid-induced leucine-zipper family related (GILR) protein capable of inhibiting apoptosis and stimulating lymphocyte activity, wherein said GILR protein contains no more than ten amino acid changes from the amino acid sequence of SEQ ID NO:2, each of said changes being either alternative conservative substitutions of amino acid residue positions selected from the group consisting of residue positions 22, 50, 75, 84, 112, 122, 127, and 128 of SEQ ID NO:2 within one of the following five groups of amino acid residues:

(1) Ala, Ser, Thr, Pro, Gly;

(2) Asp, Asn, Glu, Gln;

(3) His, Arg, Lys;

(4) Met, Leu, Ile, Val, Cys; and

(5) Phe, Tyr, Trp,

or deletion of one or more of residues 123, 124, and 125 of SEQ ID NO:2.

12. The GILR protein of claim 11, wherein said alternative conservative substitutions of amino acid residue positions are selected from the group consisting of residue positions 50, 75, 84, 122, and 128.

13. A composition for the inhibition of apoptosis in cells or for stimulating lymphocyte activation, comprising, as an active ingredient, the GILR protein of claim 11.

14. A chemically modified GILR protein of claim 11, wherein said GILR protein of claim 11 is chemically modified by being conjugated or completed with molecules facilitating or enhancing the transport of said GILR protein across cell membrane and wherein the chemically modified GILR protein has the same or higher biological activity as said GILR protein.

15. A composition for the inhibition of apoptosis in cells or for stimulating lymphocyte activation, comprising, as an active ingredient, the chemically modified GILR protein of claim 14.

* * * * *

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#13	Search **gilz cell**	11:01:36	32
#14	Search **gilz cell t**	11:01:23	19
#12	Search **GILZ tcell**	11:01:14	0
#11	Search **GILZ tcekk**	11:01:07	37
#10	Search **GILZ CD4**	11:00:52	3
#8	Search **GILZ mouse**	10:59:25	16
#7	Search **GILZ**	10:59:19	37
#3	Search **enucleated oocytes after increased**	05:12:25	35
#2	Search **enucleated oocytes after**	05:12:16	290
#1	Search **enucleate oocyte after**	05:12:13	0

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Application Number [ ] SEARCH

IDS Flag Clearance for Application 10630926

IDS Information

Content	Mailroom Date	Entry Number	IDS Review	Reviewer
M844	07-31-2003	22	☑	11-05-2004 15:37:27 cdessau
M844	06-19-2006	36	☑	06-20-2006 14:11:15 ttabb
M844	06-19-2006	42	☑	06-22-2006 12:27:51 djacobs

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#15	Search **riccardi cd8**	11:57:24	16
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#14	Search **gilz cell t**	11:01:23	19
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A New Dexamethasone-Induced Gene of the Leucine Zipper Family Protects T Lymphocytes from TCR/CD3-Activated Cell Death

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Summary

By comparing mRNA species expressed in dexamethasone (DEX)–treated and untreated murine thymocytes, we have identified a gene, glucocorticoid-induced leucine zipper (*GILZ*), encoding a new member of the leucine zipper family. *GILZ* was found expressed in normal lymphocytes from thymus, spleen, and lymph nodes, whereas low or no expression was detected in other nonlymphoid tissues, including brain, kidney, and liver. In thymocytes and peripheral T cells, *GILZ* gene expression is induced by DEX. Furthermore, *GILZ* expression selectively protects T cells from apoptosis induced by treatment with anti-CD3 monoclonal antibody but not by treatment with other apoptotic stimuli. This antiapoptotic effect correlates with inhibition of Fas and Fas ligand expression. Thus, GILZ is a candidate transcription factor involved in the regulation of apoptosis of T cells.

Introduction

Apoptosis (programmed cell death [PCD]) is involved in cell and tissue development as well as in the control of neoplastic growth (Kerr et al., 1972; Wyllie et al., 1980; Bursch et al., 1992; Cohen, 1993; Osborne and Schwartz, 1994). A number of stimuli can either induce or inhibit lymphocyte PCD through activation of molecules acting at different levels, including the cell membrane, cytoplasm, and nucleus. The definition of the signaling pathways involved in the control of apoptosis has important implications for the understanding of normal tissue development and drug resistance.

In the T cell lineage, several pathways have been identified that regulate apoptosis negatively or positively (Dent et al., 1990; MacDonald and Lees, 1990). In particular, apoptosis activated through the antigen interaction with the T cell receptor (TCR)/CD3 complex is responsible for negative selection (Jenkinson et al., 1989; Smith et al., 1989).

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Engagement of the TCR/CD3 complex, (either by antigen-presenting cells [APCs] presenting antigenic peptide or by anti-CD3 antibody), cytokines, coaccessory molecules, and tissue microenvironment triggers a series of activation events, which can contribute to regulate cell survival (Nieto and Lopez-Rivas, 1989; Nieto et al., 1990; Migliorati et al., 1993; Boise et al., 1995; Akbar and Salmon, 1997; Ayroldi et al., 1997). Elevation of intracellular Ca^{2+}, protein phosphorylation/dephosphorylation, up-regulation of the antioxidant glutathione, expression of Bcl-2/Bcl-x and of the Fas/Fas ligand (Fas/FasL) systems, and activation of transcription factors, such as NF-κB, Myc, Fos, and Jun, profoundly influence T cell apoptosis (Itoh et al., 1991; Shi et al., 1992; Smeyne et al., 1993; Veis et al., 1993; Goldstone and Lavin, 1994; Alderson et al., 1995; Dhein et al., 1995; Ju et al., 1995; Beg and Baltimore, 1996). Some of these signals can induce apoptosis in thymocytes, mature T cells, and T cell hybridomas (Webb et al., 1990). In particular, activation of T cell hybridomas leads to cell cycle arrest, followed by apoptosis. This activation-induced cell death (AICD) (Kabelitz et al., 1993) requires the interaction of Fas with FasL (Alderson et al., 1995; Dhein et al., 1995; Ju et al., 1995).

Among different stimuli, glucocorticoid hormones (GCH) are also critical regulators of T cell development (Wyllie, 1980; Cohen and Duke, 1984). In particular, dexamethasone (DEX), a synthetic GCH, which by itself induces apoptosis in T cell hybridomas and in normal T lymphocytes, can inhibit AICD induced by triggering of the TCR/CD3 complex (Zacharchuk et al., 1990). This inhibition may be due to a number of events, including prevention of activation-induced expression of FasL (Yang et al., 1995a, 1995b).

To study the role of GCH in the regulation of lymphocyte apoptosis, we attempted the isolation of genes that are induced by DEX and modulate apoptosis in T cells. We report here the identification of a gene, glucocorticoid-induced leucine zipper (*GILZ*), coding for a novel member of the leucine zipper family. Our results indicate that *GILZ* gene is induced in thymocytes and peripheral T cells by DEX. Furthermore, we show that *GILZ* expression selectively protects T cells from apoptosis induced by treatment with anti-CD3 monoclonal antibody (MAb) but not by treatment with other apoptotic stimuli, and we show that this inhibition correlates with inhibition of anti-CD3–induced up-regulation of Fas and FasL expression.

Results

Isolation of the *GILZ* cDNA

As part of a research program aimed at studying the role of glucocorticoid hormones in the regulation of lymphocyte apoptosis, we attempted the isolation of mRNA species induced by three hours of treatment with the synthetic glucocorticoid hormone DEX (100 nM) in freshly isolated thymocytes.

Comparing the cDNAs from untreated and DEX-treated cells by the subtraction probe technique, we

identified some mRNA species overexpressed in the treated cells. Upon sequencing various isolated cDNAs, one of them, *GILZ*, was shown to have some homology (67% identity in the coding region) with the mouse TSC-22, a transforming growth factor β-inducible leucine zipper gene (Shibanuma et al., 1992), and no homology with other sequences present in European Molecular Biology Laboratory and GenBank databases. This probe was used to screen a thymus lymphocyte cDNA library in order to isolate the corresponding full-length cDNA clone. Several clones were isolated, and three of them were 1972 bp long and displayed the same sequence. Since Northern blot analysis (Figure 1) indicated that *GILZ* mRNA was about 2 kb long, these clones were though to represent full-length cDNAs. This was confirmed by experiments with primer-extension technique (data not shown).

GILZ Expression in Tissues: Induction in T Lymphocytes

We performed experiments to define *GILZ* expression in different tissues. Results indicated that *GILZ* mRNA was clearly detectable by Northern blot analysis in freshly isolated thymocytes, spleen, and lymph node cells; slightly detectable in bone marrow, kidney, and lung cells; and not detectable in liver, heart, and brain cells, suggesting that the gene is mainly expressed in lymphoid tissues (Figure 1A). We then performed experiments to test the possible effect of DEX treatment on lymphoid tissue. Results indicate that *GILZ* expression was clearly increased by treatment with DEX in fresh thymocytes and lymphocytes from peripheral lymphoid tissues, including spleen and lymph nodes (Figure 1B). On the contrary, DEX treatment did not induce detectable *GILZ* expression in liver cells (data not shown).

The Protein Coded by *GILZ* Is a Leucine Zipper Protein

Nucleotide sequence of the three cDNA clones showed the presence of a single base pair open reading frame, beginning at nucleotide position 206 and extending to a tumor-associated antigen termination codon at position 617. The putative initiation codon at position 206 is surrounded by a sequence (GAACCATGA), in good agreement with the consensus sequence for initiation of translation in eukaryotes (Kozak, 1989). The termination codon is followed by a 3′ untranslated region of 1355 bp. A polyadenylation signal is present 45 bp 5′ to the poly (A) tail (Figure 2A).

The protein encoded putatively by the *GILZ* mRNA is a leucine zipper protein of 137 amino acids (aa) (Figure 2A) that displays 64% identity with TSC-22 and significant homologies with other molecules belonging to the leucine zipper family (Figure 2B; Hope and Struhl, 1987; Lamph et al., 1988; Yamamoto et al., 1988; Nicholas et al., 1991; Shibanuma et al., 1992), as confirmed by the presence of four leucine residues at positions 76, 83, 90, and 97 and one asparagine at position 87. Furthermore, a proline and acid aa rich region (PAR) is present at the 3′ end region (double underlined in Figure 2A).

The predicted molecular weight of the putative mature protein before further posttranslation modifications is

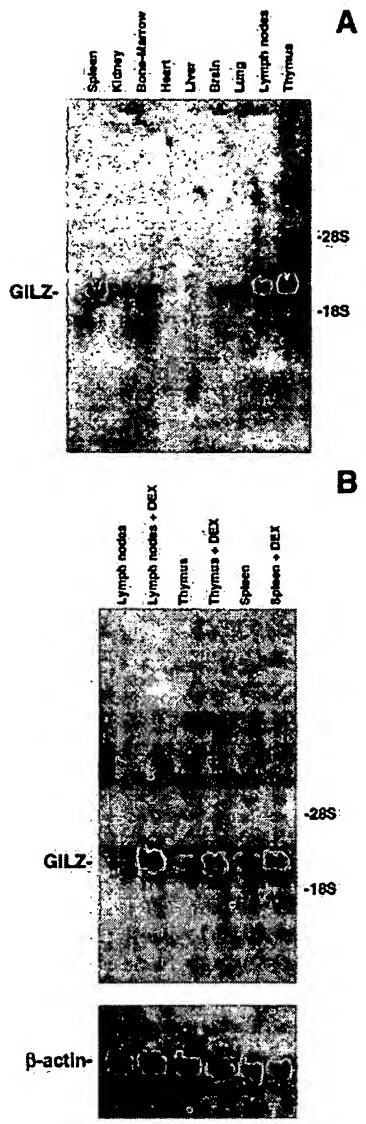

Figure 1. Northern Blot Analysis of *GILZ* Expression

(A) Organ distribution of *GILZ* mRNA. Total RNA was extracted, separated on agarose gel, and transferred to nitrocellulose filter. The filter was hybridized with nick translation labeled *GILZ* cDNA probe, washed, and exposed for autoradiography for 8 days. Each lane was loaded with 20 μg of total RNA.

(B) Effect of DEX on *GILZ* induction. Cells were either untreated or treated with 100 nM DEX for 3 hr. Total RNA (25 μg) was extracted, run on a gel, and transferred to the filter. The filter was hybridized with labeled *GILZ* cDNA and exposed for autoradiography for 24 hr.

15,165 Da. This molecular weight was confirmed by in vitro translation experiments performed using the cloned cDNA (Figure 3Aa).

A rabbit immune antiserum was used to detect a cellular product of *GILZ* in normal untreated or DEX-treated (6 hr treatment) thymocytes. In particular, a band of molecular mass of approximately 17 kDa was detected by this antiserum in the protein extract of DEX-treated thymocytes (Figure 3Ab, lane 4) and, although at a lower

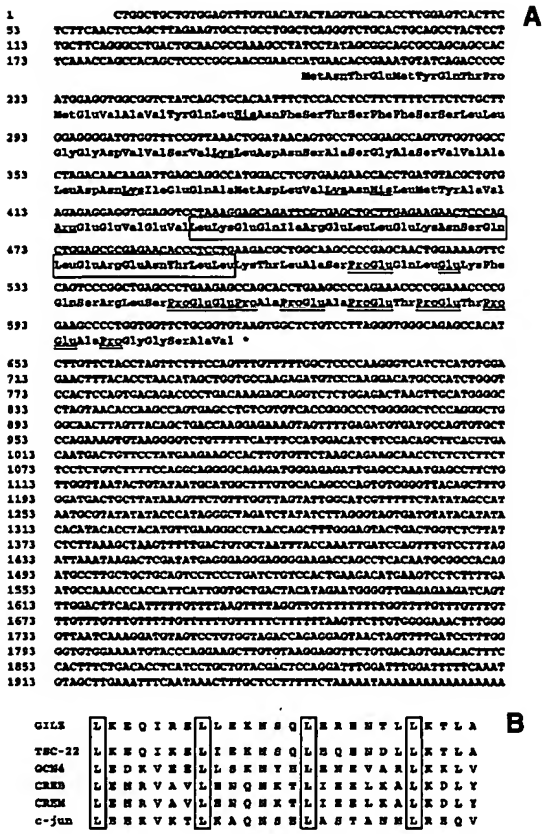

Figure 2. *GILZ* Gene Codes for a Leucine Zipper Protein

(A) Nucleotide sequence of *GILZ* and putative polypeptide sequence of GILZ. Open box, the leucine zipper domain; underlining, basic residues; double underlining, proline and acidic residues of the PAR region.

(B) Comparison of the leucine zipper motif in the open reading cDNA with those of other members of the leucine zipper family.

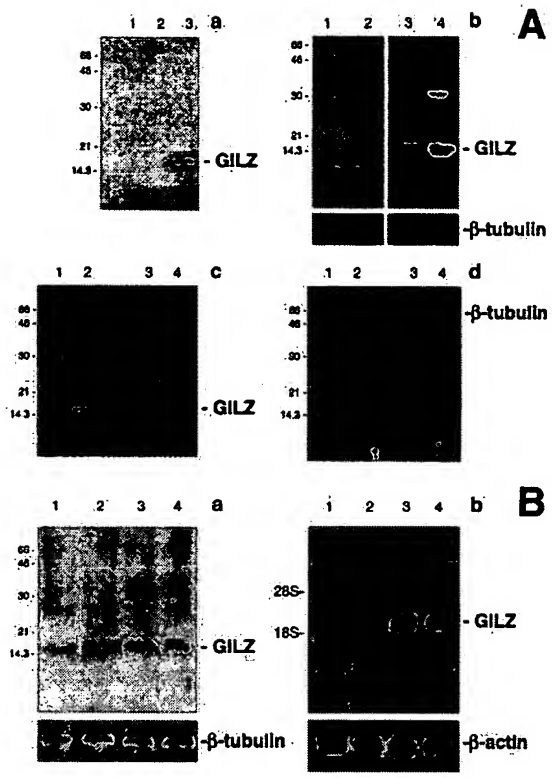

Figure 3. Analysis of GILZ Protein Expression: Subcellular Localization

(A) Product of *GILZ* cDNA. (a) In vitro translation. *GILZ* cDNA inserted into Bluescript vector was transcribed, and transcripts were translated in a rabbit reticulocyte lysate in the presence of [^{35}S]Met (lane 1, rabbit reticulocyte lysate; lane 2, empty vector; lane 3, vector with *GILZ* cDNA). (b) Western blot analysis of GILZ protein was performed by rabbit preimmune serum (lane 1, untreated thymocytes; lane 2, thymocytes treated with DEX 100 nM) or rabbit immune antiserum (lane 3, untreated thymocytes; lane 4, thymocytes treated with DEX). (c) Protein immunoblot analysis of GILZ protein in transfected 3DO clones: nucleic proteins extracted from a clone transfected with the empty vector (lane 1) or with *GILZ* cDNA (lane 2); cytoplasm proteins extracted from a clone transfected with the empty vector (lane 3) or with *GILZ* cDNA (lane 4). (d) Protein immunoblot analysis of β-tubulin protein in transfected 3DO clones: nucleic proteins from an empty vector- (lane 1) or a *GILZ*-transfected (lane 2) clone; cytoplasm proteins from an empty vector clone (lane 3) or a *GILZ*-transfected (lane 4) clone.

(B) GILZ induction by anti-CD3 plus DEX treatment. (a) Western blot analysis of GILZ protein (lane 1, untreated thymocytes; lane 2, thymocytes treated for 3 hr on plates coated with anti-CD3 (1 mg/ml); lane 3, thymocytes treated with 100 nM DEX for 3 hr; lane 4, thymocytes treated for 3 hr with anti-CD3 plus DEX. (b) Northern blot analysis of *GILZ* mRNA (lane 1, untreated thymocytes; lane 2, thymocytes treated for 3 hr on plates coated with anti-CD3 (1 μg/ml); lane 3, thymocytes treated with 100 nM DEX for 3 hr; lane 4, thymocytes treated for 3 hr with anti-CD3 plus DEX. Total RNA (25 μg) was extracted, run on a gel, and transferred to the filter. The filter was hybridized with labeled *GILZ* cDNA and exposed for autoradiography for 48 hr.

level, in the protein extract of untreated thymocytes (Figure 3Ab, lane 3) in a Western blot analysis. A rabbit preimmune serum was used as control with protein extract of untreated (Figure 3Ab, lane 1) or of DEX-treated thymocytes (Figure 3Ab, lane 2).

GILZ Expression: Subcellular Localization

We also performed experiments to define the subcellular localization of GILZ. For that purpose we evaluated the levels of GILZ in the nucleus and the cytoplasm by Western blot experiments in clones transfected with the empty vector or with the *GILZ* cDNA. Results of a representative experiment (Figures 3Ac and 3Ad) showed that GILZ is detectable in the nucleus of the *GILZ*-transfected clone (Figure 3Ac, lane 2) but not in the cytoplasm (Figure 3Ac, lane 4) or in the nucleus and cytoplasm of control clone (Figure 3Ac, lanes 1 and 3, respectively).

Anti-β-tubulin antibody was used to control the possible nuclei contamination with cytoplasm material. Results in Figure 3Ad indicate that β-tubulin is present in the cytoplasm protein extracts of control (Figure 3Ad, lane 3) and *GILZ*-transfected clones (Figure 3Ad, lane 4) but not in the nuclear extracts (Figure 3Ad, lanes 1 and 2).

We also performed experiments to evaluate whether GILZ protein (Figure 3Ba) and mRNA (Figure 3Bb) are

induced by treatment for 3 hr with anti-CD3 MAb or anti-CD3 plus DEX. Results of a representative Western blot experiment (Figure 3Ba) show that GILZ protein is not induced by treatment with anti-CD3 MAb (Figure 3Ba, lane 2) but is induced by treatment with DEX (Figure 3Ba, lane 3) or anti-CD3 plus DEX (Figure 3Ba, lane 4) in normal thymocytes. Results of a Northern blot assay (Figure 3Bb) show that *GILZ* mRNA is not induced by treatment with anti-CD3 MAb (Figure 3Bb, lane 2) but is induced by treatment with DEX (Figure 3Bb, lane 3) or anti-CD3 plus DEX (Figure 3Bb, lane 4). Similar results are obtained when spleen and lymph node cells are used and GILZ expression is evaluated at 3, 10, and 20 hr after treatment (data not shown).

GILZ Expression in Transfected T Cells Confers Resistance to TCR/CD3-Induced Apoptosis but Not to Apostosis Induced by Other Stimuli

To test the possible effects of *GILZ* expression on apoptosis, we transfected a hybridoma T cell line, 3DO, which does not express detectable *GILZ* and has been used widely in the investigation of apoptosis induced by anti-CD3 antibodies (Ayroldi et al. 1995; Vito et al., 1996), with an expression vector in which the *GILZ* cDNA is expressed under the control of the cytomegalovirus promoter. The apoptosis induced by anti-CD3 MAb in 3DO cells has been shown previously to be dependent from the Fas/FasL system (Ayroldi et al., 1997). We also transfected the empty vector as a control (pcDNA3 control). After selection with G418 antibiotic, cell clones were screened for *GILZ* expression by RNase protection analysis (Figure 4A). For each transfection, nine clones were tested and used for functional characterization. In addition, six normal untransfected clones (nuc/1–6) were tested as additional controls (Figure 4B).

The results showed that all cell clones overexpressing *GILZ* (clones *GILZ*/1–9) were resistant variably to anti-CD3 MAb–induced apoptosis (apoptosis between 5% and 10%) when compared to pcDNA3 control clones (clones pcDNA3/1–9, apoptosis between 45% and 60%) with $P<0.001$. No significant differences between pcDNA3 clones and untransfected clones (clones nuc/1–6, apoptosis between 45% and 60%) were detectable (Figure 4B). To exclude a possible effect of a *GILZ* gene on TCR/CD3 membrane expression, which could by itself account for diminished sensitivity to anti-CD3–induced apoptosis, all clones were stained with anti-CD3 MAb and analyzed by flow cytometry. No differences in CD3 expression were detected between transfected and untransfected clones (data not shown).

It has been shown that T cell apoptosis can be induced by various stimuli other than triggering of the TCR/CD3 complex, including corticosteroids, radiation, and starvation (Wyllie, 1980; Bansal et al., 1991; Lowe et al., 1993). We performed experiments to test whether *GILZ* expression inhibits T cell apoptosis induced by other stimuli. Results obtained with the clones *GILZ*/1,5,7 and pcDNA3/4,7,8 (Figure 5) indicate that *GILZ* overexpression does not counteract apoptosis induced by DEX, various doses of ultraviolet (UV) irradiation, starvation, or triggering by cross-linked anti-Fas MAb (Fas is normally

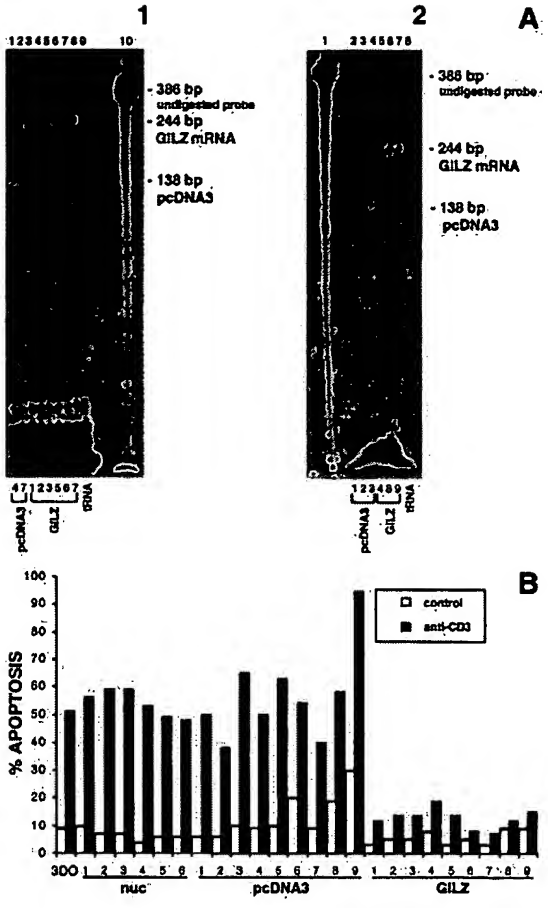

Figure 4. *GILZ* Expression Inhibits TCR/CD3-Activated Apostosis

(A) RNase protection analysis of *GILZ* mRNA expression in transfected clones. (1) Clones transfected with empty pcDNA3 (lanes 1 and 2) or with *GILZ* cDNA (lanes 3–8); tRNA (lane 9); undigested probe (lane 10). (2) Undigested probe (lane 1); clones transfected with empty pcDNA3 (lanes 2–4) or with *GILZ* cDNA (lanes 5–7); tRNA (lane 8). Twenty micrograms of RNA was loaded on each lane. The protected antisense mRNA fragment in clones transfected with empty pcDNA3 or with *GILZ* cDNA is of 138 and 244 bp, respectively. (B) Protection of transfected 3DO clones from TCR-induced death. 3DO cells were transfected by electroporation with 15 μg linearized pcDNA3 or 15 μg linearized pcDNA3 vector expressing the *GILZ* cDNA. After 36 hr from transfection, the cells were cultured in medium containing G418 0.8 mg/gr active form, and 100 μl/ of cell suspension were plated in 96-wells plates (4 for each transfection). For induction of apoptosis, cells were cultured 20 hr on plates coated with anti-CD3 (1 μg/ml). The percentage of cell death was assessed by measurement of the DNA content of isolated nuclei stained with propidium iodide. The data shown are representative of three independent experiments.

expressed in 3DO cells; Figure 6A and Ayroldi et al., 1997). These results suggest that *GILZ* can modulate apoptosis induced by triggering of a TCR/CD3 complex but not by other stimuli.

Expression of Fas and FasL in *GILZ*-Transfected T Cells

It has been suggested that T cell AICD is also dependent on Fas/FasL interaction (Alderson et al., 1995; Dhein et

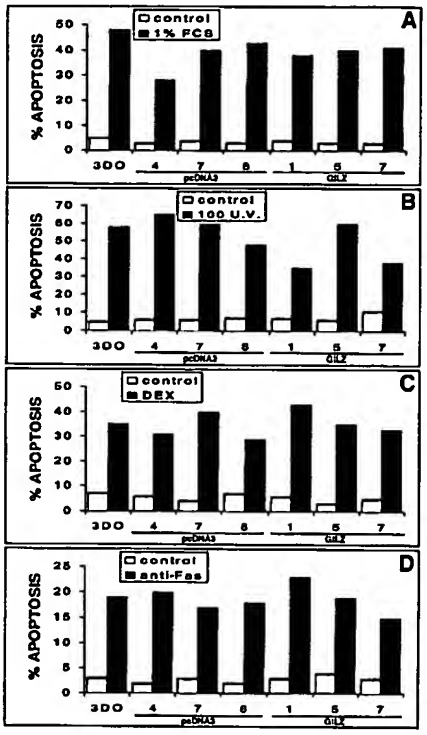

Figure 5. Analysis of Apoptosis Induced by Other Stimuli on Transfected 3DO Clones

(A) Withdrawn trophic factor. (B) UV irradiation (100 J/m2). (C) DEX treatment (100 nM). (D) anti-Fas MAb (5 μg/ml). All groups were treated for 20 hr. Cell death was measured as indicated in Figure 4B.

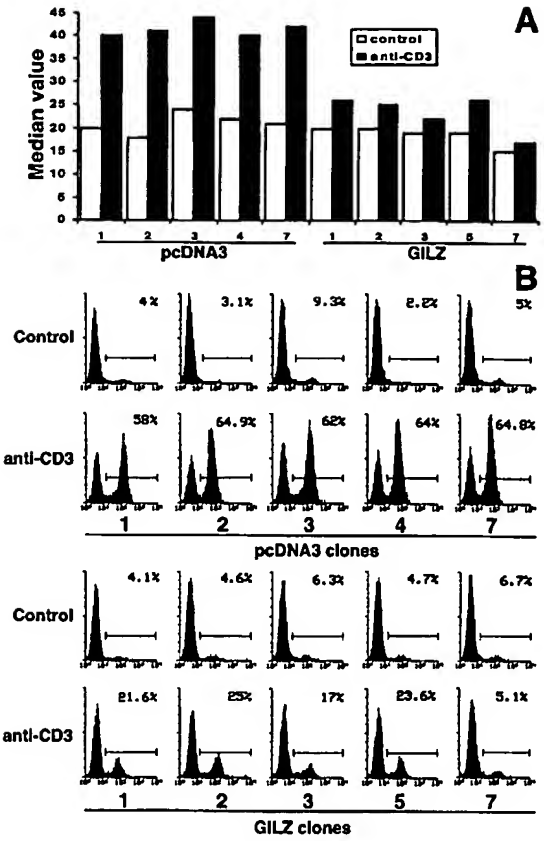

Figure 6. Fas and FasL Expression on Transfected 3DO Clones

3DO cells transfected with empty vector or with *GILZ* pcDNA3 were triggered with anti-CD3 MAb (1 μg/ml) for 20 hr and then analyzed by a fluorescence-activated cell sorter.

(A) Fas expression. Results (mean of 3 experiments) are expressed as values of the histogram median. A PE-hamster IgG (isotype-matched MAb) was used to calculate the background. The standard errors (<10%) are omitted for clarity.

(B) FasL expresssion. Percentage of positive cells is indicated in each histogram.

al., 1995; Ju et al., 1995). In particular, we have shown previously that anti-CD3–induced apoptosis in 3DO cells is blocked by soluble anti-Fas MAb, whereas cross-linked anti-Fas MAb induces cell death directly (Ayroldi et al., 1997). We performed experiments to test whether blocking of Fas (using soluble, non–cross-linked anti-Fas MAb, 1 μg/ml) could also inhibit the anti-CD3–induced apoptosis in 3DO clones. Results indicate that blocking of Fas significantly inhibits CD3-induced cell death (apoptosis, mean of results obtained with three normal clones in a 20 hr assay: 4 ± 1 in untreated controls, 63 ± 5 in anti-CD3-treated, and 29 ± 6 in clones treated with anti-CD3 plus soluble anti-Fas; $P<0.01$ comparing anti-CD3-treated with anti-CD3- plus anti-Fas-treated clones). We performed experiments to assess whether the inhibition of apoptosis in *GILZ*-transfected cells could be mediated by an effect on Fas/FasL system expression. Results show that 20 hr anti-CD3 MAb treatment induced augmentation of Fas (Fas is already detectable in untreated 3DO cells; Figure 6A) and induction of FasL (Figure 6B) expression in clones transfected by the empty vector (pcDNA3/1,2,3,4,7), but it did not augment Fas and FasL expression in clones overexpressing *GILZ* (clones *GILZ*/1,2,3,5,7; Figure 6). Similar results were obtained when the expression of mRNA of Fas (Figure 7A) and FasL (Figure 7B) was evaluated. These data indicate that *GILZ*-mediated inhibition of anti-CD3–induced apoptosis is the result of inhibition of Fas and FasL mRNA transcription.

We also performed kinetic experiments to evaluate better the FasL expression and the induction of apoptosis. Results of a representative experiment indicate that while in an empty vector-transfected clone, FasL expression was evident 10 hr after anti-CD3 treatment; when the apoptosis was also detectable, neither FasL expression nor apoptosis was induced in an anti-CD3–treated *GILZ*-transfected clone (Table I). These results indicate further that *GILZ* expression inhibits TCR/CD3-activated apoptosis and FasL expression.

Discussion

We performed studies in the attempt to identify genes whose transcription is regulated by DEX treatment that could be involved in the modulation of T lymphocyte apoptosis. The data describe the isolation of a new

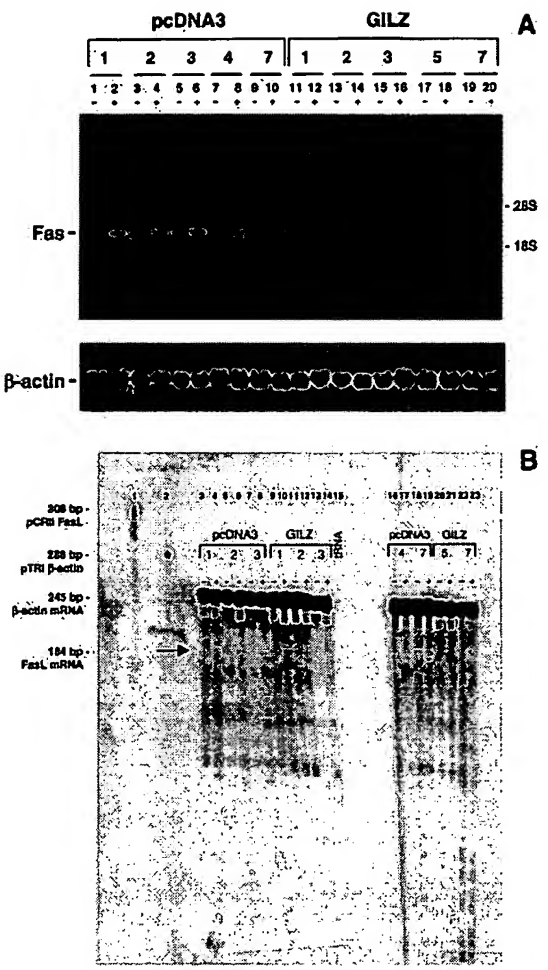

Figure 7. Effect of *GILZ* on Expression of Fas and FasL mRNA

(A) Expression of Fas mRNA in transfected clones. Total RNA was extracted, separated on agarose gel, and transferred to nitrocellulose filter. The filter was hybridized with nick translation labeled *GILZ* cDNA probe, washed, and exposed for autoradiography for 24 hr. Each lane was loaded with 20 μg of total RNA. Clones transfected with empty pcDNA3 untreated (lanes 1, 3, 5, 7, 9) or treated with anti-CD3 MAb (1 μg/ml) for 20 hr (lanes 2, 4, 6, 8, 10); clones transfected with *GILZ* cDNA untreated (lanes 11, 13, 15, 17, 19) or treated with anti-CD3 MAb (1 μg/ml) for 20 hr (lanes 12, 14, 16, 18, 20).

(B) RNase protection analysis of FasL mRNA expression in the transfected clones. FasL (lane 1) or β-actin (lane 2) undigested probes; clones transfected with empty pcDNA3 untreated (lanes 3, 5, 7, 16, 18) or treated with anti-CD3 MAb (1 μg/ml) for 20 hr (lanes 4, 6, 8, 17, 19); clones transfected with *GILZ* cDNA untreated (lanes 9, 11, 13, 20, 22) or treated with anti-CD3 MAb (1 μg/ml) for 20 hr (lanes 10, 12, 14, 21, 23); tRNA (lane 15). 20 μg RNA was loaded on each lane. The protected antisense mRNA FasL fragment is 184 bp.

member of the leucine zipper family, GILZ, able to inhibit TCR-activated cell death.

The GILZ putative protein has good homology with all the other members of this family in the leucine zipper domain including the TSC-22, whose function has not been defined and has also been shown to be induced by DEX treatment (Shibanuma et al., 1992). Four leucine residues spanned by 7 aa (at positions 76, 83, 90, and 97) and an asparagine residue (at position 87) within the leucine zipper domain (Figure 2) are compatible with the canonical leucine zipper structure of the family. GILZ, like TSC-22, also contains a PAR (Drolet et al., 1991), and a noncanonical basic domain (6 basic aa underlined in Figure 2A) that could be involved in binding to DNA (Hurst H.C., 1994; Takashi and Schultz, 1996). Moreover, contrary to other leucine zipper molecules (Hope and Struhl, 1987; Yamamoto et al., 1988; Nicholas et al., 1991; Goldstone and Lavin, 1994), both TSC-22 and GILZ have relatively small dimensions (143 and 137 aa, respectively) suggesting that these two may represent a new family of low-molecular-weight leucine zipper proteins.

The *GILZ* mRNA is clearly detectable by Northern blotting in freshly isolated thymocytes, spleen, and lymph node cells, and mRNA and protein expression is increased in lymphoid tissues, such as thymocytes, spleen, and lymph nodes by treatment with DEX (Figures 1 and 3). Although these results may suggest that this gene is mainly expressed in T lymphocytes, a similar expression in other tissues (including those in which we have found low or no mRNA expression, Figure 1) cannot be excluded based on our present results. For instance, *GILZ* gene induction may occur as a result of inflammatory processes and tissue regeneration or in the presence of tissue-specific signals.

GILZ expression is not induced by treatment with anti-CD3 MAb. However, the increase in *GILZ* expression following DEX/T cell interaction suggests that this gene may be involved in regulating lymphocyte death. DEX has been shown previously to antagonize apoptosis in T lymphocytes, including thymocytes and T cell hybridomas, activated by triggering of the TCR/CD3 complex (Zacharchuk et al., 1990; Yang et al., 1995a). Moreover, it has been shown that transgenic expression of antisense RNA to glucocorticoid receptor significantly affects the thymocyte development (King et al., 1995). The results, obtained by transfection experiments, indicate that the *GILZ* gene is able to inhibit T cell apoptosis induced by treatment with anti-CD3 MAb. On the contrary, the same transfected clones are not protected against the PCD induced with other typical apoptotic agents, such as DEX, UV irradiation, serum starvation, or triggering of Fas by cross-linked anti-Fas MAb. These data indicate that GILZ is specific in counteracting T cell death activated by triggering of the TCR/CD3 complex and suggest that GILZ could contribute in part to the DEX-induced inhibition of TCR/CD3-activated apoptosis and to the regulation of T cell development.

This protective effect opens the question about the possible mechanism(s) of *GILZ*-induced inhibition of apoptosis. Our present results indicate that the apoptosis inhibition, associated to *GILZ* overexpression, correlates with the inhibition of Fas and FasL up-regulation induced by treatment with anti-CD3 MAb (Figures 6, 7, and 8). These data are in part compatible with previous observations showing that DEX can inhibit FasL expression thus regulating AICD (Yang et al., 1995a, 1995b). One possibility is that GILZ interacts with other molecules, at the present unknown, which are involved in the activation of Fas and FasL genes. GILZ

Table 1. Time Course of FasL Expression Related to Anti-CD3–Induced Apoptosis on an Empty Vector–or *GILZ*-Transfected Clone[a]

	3 hr		6 hr		10 hr	
	FasL	Apoptosis	FasL	Apoptosis	FasL	Apoptosis
pcDNA3	0.7[b]	1.3	1.0	2.5	5.7	2.9
pcDNA3 +Anti-CD3	1.2	1.7	1.1	3.7	81.1	72.0
GILZ	1.0	0.3	1.7	1.8	0.1	2.8
GILZ + Anti-CD3	0.6	0.9	1.2	1.4	1.4	2.7

[a] An empty vector–transfected clone (pcDNA3, clone 4) and a *GILZ*-transfected clone (*GILZ*, clone 7) were used in the experiment.
[b] Numbers represent the percentage of cells positive for FasL as determined by flow cytometry analysis using anti-FasL mAb or the percentage apoptosis as determined by flow cytometry analysis of propidium iodide-labeled cells.

could interact either with signal(s) induced by TCR/CD3 triggering in activated lymphocyte or directly with transcription factors involved in the regulation of Fas and FasL gene transcription acting as a suppressor factor. Our results, showing that GILZ is present in the cell nucleus and that its overexpression inhibits the Fas and FasL mRNAs, suggest but do not prove that GILZ mediates inhibition of apoptosis acting as a transcriptional repressor. Experiments using inducible transfectants could better address this point and further clarify the role of *GILZ* expression in the inhibition of Fas and FasL mRNA transcription. Such studies are currently being pursued in our laboratory.

Experimental Procedures

Cells and Culture Conditions

Cells of different organs were obtained from 3- to 5-week-old C3H/HeN mice purchased from Charles River (Milan, Italy). Lymph nodes and splenocytes were passed through a nylon wool column for removal of adherent cells. The cell suspensions were washed, filtered, and adjusted to a concentration of 8×10^6 cells/ml in complete medium. The cells were incubated at 37°C alone or with 100 nM DEX (Sigma, St. Louis, MO) for different times.

A subline obtained in our laboratory of the mouse hybridoma T cell line (3DO) maintained in suspension in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 10 μM HEPES buffer was used for transfection experiments.

RNA Preparation

Total cytoplasm RNA was isolated by using the protocol of Chirgwin (1979). Polyadenylated RNA was obtained as described previously (Maniatis et al., 1989).

Library Construction

A directionally cloned cDNA library was constructed by using polyadenylated cytoplasm RNA from thymocytes cultured for 3 hr in the presence of DEX, according to the protocol of Maniatis et al. (1989). First-strand cDNA was obtained with a reverse reaction using an oligo (dT) primer (10 μg) and 7 μg of polyadenylated RNA. To monitor synthesis, 20 μCi [^{32}P]dCTP (3,000 Ci/mmol) was included in the reaction mixture. Second-strand cDNA was synthesized according to the procedures described by Gubler and Hoffman (1983). The cDNA was blunt-ended by using T4 polymerase (Boehringer Mannheim, Mannheim, Germany) and then methylated with EcoRI methylase (Boehringer Mannheim). EcoRI linkers were ligated to cDNA with T4 DNA ligase (New England Biolabs, Beverly, MA) at 16°C for 12 hr. Following the ligation of linkers, the reaction was inactivated warming at 68°C for 15 min. The cDNA suspension was precipitated in ethanol and purified on the column of CL4B (Invitrogen BV, San Diego, CA).

The cDNA was inserted into λgt11 arms using EcoRI adaptors, following manufacture's protocol (Invitrogen BV). Recombinants clones (0.25×10^4 plaque-forming units/μl) were screened by hybridization with the subtraction probe.

Subtraction Probe Procedure and Library Screening

To construct the subtracted probe, a biotinylated copy of the uninduced pool of mRNA (10 μg) and ^{32}P-labeled cDNA from the induced mRNA (1 μg) were coprecipitated in ethanol. The precipitate was dried and dissolved in 2× hybridization buffer. Sample was heated at 100°C for 1 min and then incubated at 68°C for 24 hr. To separate unhybridized from hybridized sequences, reaction was diluted 10 to 15 times with streptavidin binding buffer and incubated with streptavidin for 10 min at room temperature. Two phenol-chloroform extraction were performed. After precipitation, the labeled cDNA probe was resuspended in 50 μl of sterile water and used directly as a subtraction probe for screening the cDNA library.

Nitrocellulose filters (Amersham Life Science International PLC, Beckinghamshire, England) obtained by blotting plates containing 5×10^4 clones were hybridized in 5× SSC, 5× Denhardt's solution, 1% SDS, 100 μg/μl tRNA (Sigma), and 20 mM sodium pyrophosphate (pH 6.8) at 42°C for 12 hr, and the final wash was in 0.2× SSC, 0.1% SDS at 65°C for 30 min.

Northern Blot Analysis

Indicated amounts (see figure legends) of total cytoplasm of RNA were separated in 1.2% agarose gels and transferred to nitrocellulose filters (Scheicher and Schuell, Dassel, Germany). DNA probes were ^{32}P-labeled using the nick translation kit from Boehringer Mannheim. Hybridization was carried out overnight. Filters were washed three times in 0.2× SSC with 0.5% SDS at 37°C followed by two washes at 65°C.

Primer-Extension Technique

The primer extension has been performed according to the procedure of Maniatis et al. (1989). The radiolabeled DNA primer (10^5 cycles per minute), complementary to sequence from nucleotide at position 298 to nucleotide at position 327 of the *GILZ* gene, was mixed with 20 μg of mRNA from DEX-treated (3 hr) thymocytes.

DNA Sequence Determination

cDNA clones were sequenced using T_7 DNA polymerase (Sequenase kit, US Biochemical, Cleveland, OH) in conjunction with custom-synthesized 20- and 21-mer oligonucleotide primers (complementary to the cDNA sequence) and primers complementary to the plasmid-cloning site sequences. Overlapping sequences were obtained for both strands of the cDNA. cDNA sequences were derived from clones isolated from the screening of cDNA library.

All sequences analyzed and identification of structural motifs were done with the Wisconsin Sequence Analysis Package Software Program (Genetics Computer Group, University Research Park, Madison, WI).

In Vitro Translation

RNA was translated in vitro using a rabbit reticulocyte lysate (Promega Corporation, Madison, WI) by the manufacturer's recommended procedure in the presence of [^{35}S]methionine (Amersham), and the products were analyzed with 15% SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gel was fixed and autoradiographed.

Preparation of Rabbit Anti-Mouse Antiserum and Western Blot Analysis

A rabbit polyclonal antiserum recognizing *GILZ* was prepared with the use of a fusion protein containing the full GILZ amino acid sequence fused to glutathione S-transferase (Pharmacia, Upsala, Sweden). Glutathione S-transferase fusion protein was expressed in Escherichia coli, induced with 1 mM isopropyl-β-D-thiogalactopyransside and purified with glutathione (GSH)-agarose beads as described previously (Tan et al., 1994). This preparation was used to immunize New Zealand white rabbits (1 mg/rabbit). After 4 weeks a booster injection of 0.2 mg of protein was given intravenously and blood was collected 1 week later for preparation of antiserum. The antiserum was purified using fusion protein immobilized on nitrocellulose filter according to Maniatis protocol. The antiserum was used for Western blot analysis of proteins extracted from thymocytes treated with or without DEX and of proteins extracted from 3DO clones, as previously described (Ayroldi et al., 1997). Nuclear proteins were prepared from isolated nuclei as described previously by others (Marzluff and Huang, 1985), and an anti-β-tubulin MAb (Calbiochem, San Diego, CA) was used as control efficacy of nuclei separation.

Transfections of Cultured Cells

The *GILZ* cDNA coding sequence (874 bp) was cloned into pcDNA3 plasmid (Invitrogen) for expression in mammalian cells. 3DO cells were transfected by electroporation (300 mA, 960 mF) with 15 μg of linearized pcDNA3 vector (control clones) or 15 μg of linearized pcDNA3 vector expressing the *GILZ* cDNA. Thirty-six hours after transfection, the cells were cultured in medium containing G418 0.8 mg/g active form (GIBCO-BRL, Life Technologies, Paisley, Scotland), and 100 μl/ml of cell suspension was plated in 96-wells plates (4 for each transfection). After 15–20 days, no more than 15% of the wells presented live growing cells. These cells were considered clones and then analyzed in RNAse protection for the expression of exogenous *GILZ* (Vito et al., 1996; Nocentini et al., 1997).

RNase Protection Analysis

GILZ probe for RNase protection analysis was constructed by polymerase chain reaction (PCR) using the forward primer CCATCT GGGTCCACTCCAGT (located on *GILZ*, 763-782 bp) and the Reverse primer AGGACAGTGGGAGTGGCACC (located on pcDNA3). FasL probe for RNase protection analysis was constructed by PCR using the forward primer CACATATGGAACCGCTCTGATC and the reverse primer CATTAGCACCAGATCCTCAGGA (located on FasL cDNA, 610–631 bp and 722–743, respectively). PCR products (244 bp for *GILZ* and 184 for FasL) were cloned into pCRII vector using the TA Cloning kit (Invitrogen). The cloning product were sequenced to exclude point mutation. Linearized β-actin probe was purchased from Ambion (Austin, TX). *GILZ* probe, digested with XbaI and KpnI (New England Biolabs), was subcloned into a pGEM-3 vector and was linearized with XbaI (Boehringer Mannheim), while FasL probe was linearized with BamHI (Boehringer Mannheim). Plasmid DNA was transcribed with T_7 RNA polymerase (GIBCO-BRL) in the presence of 50 μM [α^{32}P]UTP. Following gel purification, 2×10^5 counts per minute probes were hybridized to total RNA (20 μg) overnight at 60°C. RNase digestion was performed by using a RNase A (Boehringer Mannheim) (40 μg/ml) and RNase T_1 (1.5 U/μl, GIBCO-BRL) solution at 37°C for 15 min. The undigested products were treated with phenol-chloroform, precipitated with ethanol and loaded on a denaturing polyacrylamide sequencing gel. Autoradiographic exposure was carried out for 2 days.

Antibody Cross-Linking and Cell Treatment

Hamster anti-mouse CD3ε (clone 145-2C11; Pharmingen, San Diego, CA) MAb at 1 μg/ml was allowed to adhere in flat-bottomed, high-binding 96-well plates (Costar, Cambridge, MA) at 4°C in 100 μl phosphate-buffered saline. After 20 hr, plates coated with MAb were washed and transfected clones were plated at 1×10^5 cells/well and incubated at 37°C for 20 hr. Isotype-matched rat anti-mouse IgG 2b MAbs (clone R 35-38, Pharmingen) were used as a control (Ayroldi et al., 1997; Migliorati et al., 1993).

To evaluate Fas-mediated killing, 3DO cells (1×10^6) were incubated at room temperature for 30 min with 10 μg/ml of the antibody to Fas (hamster anti-mouse, clone Jo2; Pharmingen), then washed and plated on wells coated with an antibody to hamster IgG (5 μg/well; Pharmingen).

UV Irradiation, DEX Treatment, and Starvation

In some experiments clones transfected with empty pcDNA3 or *GILZ*-cDNA were exposed to different doses of UV rays from a UV Stratalinker (model 1800, Stratagene, La Jolla, CA).

Aliquots of 2 ml of transfected clones (1×10^6 cells/ml) were incubated with DEX or in deprivation conditions (1% FCS). The apoptosis was evaluated after 20 hr as described below.

Flow Cytometry Analysis

A single suspension (1×10^6 cells/sample) was incubated for 30 min on ice in 50 ml staining buffer (phosphate-buffered saline plus 5% FCS), containing 10 μg/ml hamster anti-mouse Fas MAb directly conjugated to R-phycoerythrin (PE) or PE-hamster IgG (isotype control). Both MAbs were purchased from Pharmingen. Cells were also stained with rabbit polyclonal antibody raised against a peptide corresponding to amino acids 260–279 mapping at the carboxy terminus of human FasL (Santa Cruz Biotechnology, Santa Cruz, CA) or with isotype-matched antibody, and with anti-rabbit IgG conjugated with fluorescein, F(ab')$_2$ fragment (Sigma), as a second-step reagent.

All clones were stained with hamster anti-mouse αCD3, directly conjugated with fluorescein (Pharmingen). The median or percentage of Fas and FasL histograms was calculated using lysis II research software (Becton-Dickinson, Mountain View, CA).

Apoptosis Evaluation by Propidium Iodide Solution

Apoptosis was measured by flow cytometry as described elsewhere (Nicoletti et al., 1991). Briefly, cells were centrifuged and the pellets resuspended in 1.5 ml hypotonic propidium iodide solution. The tubes were kept at 4°C in the dark overnight. The propidium iodide-fluorescence of individual nuclei was measured by flow cytometry with standard FACScan equipment (Becton Dickinson).

Statistical Analysis

Each experiment was performed at least three times. Representative experiments are shown, unless otherwise indicated in the figure legends. The means ± SD of three different experiments are instead included in the text. Because of the abnormal distribution of the data, nonparametric tests (Kruskall-Wallis's analysis of variance) were adopted for statistical evaluation.

Acknowledgments

The authors wish to express their gratitude to Dr. Riccardo Dalla Favera for providing helpful suggestions and critical comments. This work was supported by AIRC, Milan and Progetto Strategico "Ciclo cellulare e apoptosi" CNR, Rome, Italy.

Received July 7, 1997; revised September 29, 1997.

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GenBank Accession Number

The GenBank accession number for the sequence reported in this paper is AF024519.